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Total body irradiation of donors can alter the course of tolerance and induce acute rejection in a spontaneous tolerance rat liver transplantation model

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Liver transplantation is an established therapy for end-stage liver diseases. Graft rejection occurs unless the recipient receives immunosuppression after transplantation. This study aimed to explore the mechanism of acute rejection of liver allografts in rats pre-treated with total body irradiation to eliminate passenger lymphocytes and to define the role of $CD4^+CD25^+$ regulatory T cells in the induction of immunotolerance in the recipient. Male Lewis rats were used as donors and male DA rats were recipients. Rats were randomly assigned to the following four groups: control group, homogeneity liver transplantation group, idio-immunotolerance group and acute rejection group. After transplantation, the survival time of each group, serum alanine aminotransferase, total bilirubin levels, number of $Foxp3^+CD4^+CD25^+$ regulatory T cells, expression of glucocorticoid-induced tumor necrosis factor receptor on T cell subgroups, histopathology of the hepatic graft and spleen cytotoxic T lymphocyte lytic activity were measured. In the acute rejection group, where donors were preconditioned with total body irradiation before liver transplantation, all recipients died between day 17 and day 21. On day 14, serum alanine aminotransferase increased significantly to (459.2±76.9) U L^{-1}, total bilirubin increased to (124.1±33.7) μmol L^{-1} (P<0.05) and the ratio of $Foxp3^+CD4^+CD25^+$ regulatory T cells decreased significantly to 1.50%±0.50% (P<0.05) compared with the other groups. Analysis of the T cell subpopulations in the acute rejection group varied from the other groups. Histological analysis showed typical changes of acute rejection in the acute rejection group only. Preconditioning of the donors with total body irradiation eliminated passenger lymphocytes of the liver graft, and thus affected the course of tolerance and induced acute rejection after liver transplantation.

liver transplantation, immune tolerance, $Foxp3^+$, regulatory T cell, GITR

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In end-stage liver disease, liver transplantation is often performed. One disadvantage of transplantation is that graft rejection occurs unless the recipient receives immunosuppression after transplantation. The liver is an immunologically privileged organ with a capability for immunotolerance. Tolerance can be induced in some liver recipients after standard immunosuppression treatments. Previous stud-

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ies on combined liver and kidney, or combined liver and intestine transplantation have shown that liver grafts confer immunoprotection to the kidney or intestinal grafts. However, the mechanism of hepatic immunologic privilege is unknown [1]. Immunotolerance can be induced without immunosuppression in a mouse liver transplant model even when the transplant is fully major histocompatibility mismatched. Total body irradiation (TBI) is a form of radiotherapy used primarily as part of the preparative regimen for haematopoietic stem cell (or bone marrow) transplantation. TBI in a bone marrow transplantation setting serves to destroy or suppress the recipient's immune system, thereby preventing immunologic rejection of transplanted donor bone marrow or blood stem cells. However, tolerance can be prevented by TBI of the donor before liver transplantation, although the mechanism for this process is unknown [2]. This study used Lewis rats as donors and DA rats as recipients to establish spontaneous immunotolerance and acute rejection in a rat orthotopic liver transplantation model. The aims of this study were to investigate the mechanism of acute rejection induced by elimination of passenger lymphocytes in the donor liver grafts by TBI, to define the role of $CD4^+CD25^+$ regulatory T cells (Tregs) in the induction of immunotolerance after liver transplantation, and to explore the mechanism of the immunologically privileged status of the liver.

$CD4^+CD25^+$ Tregs have attracted considerable attention because of their suppressive effects on many kinds of immune cells and their crucial roles in innate and adaptive immunity. The transcription factor forkhead box P3 (Foxp3) is an intracellular marker for Tregs, and expression of this protein is important for the proliferation of Tregs and for their suppressive effects. Tregs from Foxp3 knockout mice have a reduced proliferation capability and acquire autoimmune diseases with multi-organ involvement [3]. Expression of glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR), a TNF receptor superfamily member, provides costimulatory signals that activate T cells and counteracts the suppressive activity of $CD4^+CD25^+$ Tregs. This induces an up-regulation of the immune response, thus intensifying the generation and development of autoimmune diseases [4,5]. The complicated interactions between GITR and Tregs are of interest and suggested the investigation of immunologic regulation in liver transplantation. Thus, we studied the mechanism of TBI-induced acute rejection of rat liver transplantation and the role of $CD4^+CD25^+$ Tregs in inducing immune tolerance.

1 Materials and methods

1.1 Experimental animals

Male Lewis (RT-1^1) rats weighing 200–250 g (Weitonglihua Biotechnology Company, Beijing, China) were used as donors and as recipients in some animal groups. Male DA (RT-1^a) rats weighing 250–300 g (Haerbin Experimental Animal Center, Haerbin, China) were used as recipients. All rats were bred and maintained in the animal facilities at Nanjing Medical University under specific pathogen free conditions in accordance with the guidelines of Nanjing Medical University. No immunosuppression was given to the recipient rats.

1.2 Allogeneic orthotopic liver transplantation in rats

Orthotopic liver transplantation was performed following the improved Kamada method as previously described [6]. Rats that died within 5 d of transplantation were considered operative deaths and were not included in the study.

1.3 Preconditioning with total body irradiation

TBI was performed using a linear accelerator (MD2, Siemens, Germany) under the following settings: 10 MVX ray, 1000 rad dose, and 100 cm source-skin distance [7]. Donor rats were irradiated 24 h before liver transplantation in separate plexiglass boxes, each with a 2 cm thick surround and 1.5 cm thick top and bottom walls.

1.4 Experimental design

Rats were randomly divided into 4 groups, with 12 donors and 12 recipients in each group as follows: (i) control group were Lewis rats in which no liver transplantation and TBI were performed; (ii) homogeneity liver transplantation group in which both donors and recipients were Lewis rats, and liver transplantations were performed with no special handling before operations; (iii) idio-immunotolerance group [8] in which the donors were Lewis rats and the recipients were DA rats, and liver transplantations were performed with no special handling before operations; (iv) acute rejection group [8] in which the donors were Lewis rats and the recipients were DA rats, and liver transplantations were performed after the donors were preconditioned with TBI 24 h before the surgical operation. The recipients were observed and evaluated for survival after surgery.

Peripheral blood from 6 rats in each group was obtained on days 4 and 7 after surgery by tail cutting, and the rats were sacrificed to obtain peripheral blood, liver and spleen on day 14 after surgery.

1.5 Assessment of liver function

Blood samples were obtained from the tail vein. The serum concentrations of alanine transaminase (ALT) and total bilirubin (TB) were measured with an automatic biochemistry analyzer (UVIDEC-77, Japan).

1.6 Analysis of T cell subpopulations

To 50 μL of none-stimulated peripheral blood obtained

from each recipient or control rat, 9 μL of PerCp-conjugated anti-rat CD3 monoclonal antibody (mAb) (Beckman Coulter Inc, Brea, CA, USA), 9 μL of FITC-conjugated anti-rat CD4 mAb (Beckman Coulter Inc.) and 9 μL of APC-Cy7-conjugated anti-rat CD8 mAb (Beckman Coulter Inc) were added. After haematolysis by lysis buffer and incubation in the dark at 4°C for 30 min, the blood was washed and resuspended in PBS. Then the blood cells were counted by FACSCanto (Becton Dickinson, Franklin Lakes, NJ, USA). The data were analyzed using a Beckman Coulter FACSDiva (Beckman Coulter Inc.).

1.7 Analysis of $Foxp3^+CD4^+CD25^+$ Treg

To 50 μL of none-stimulated peripheral blood obtained from each recipient or control rats, 10 μL of anti-CD4-FITC (Beckman Coulter Inc) and 10 μL of anti-CD25-APC mAbs (Beckman Coulter Inc) were added and the blood was incubated in the dark at 4°C for 30 min. After haematolysis and washing, intracellular staining was performed on $CD4^+CD25^+$ T cells using 1 mL Fixation/Perm buffer solution for 45 min. The cells were then washed and 1 μL of γ-Globulin was added to block the cells at 4°C for 15 min. The cells were then stained with 10 μL of PE-conjugated anti-rat Foxp3 mAb (Beckman Coulter Inc) or 10 μL of PE-IgG2b for isotype-control (provided by the human regulatory T cell kit, eBioscience company), in the dark at 4°C for 30 min. After washing twice, the cells were resuspended in 300 μL flow cytometry staining buffer, and were counted using FACSCanto. The data were subsequently analyzed using FACSDiva to calculate the percentage of $Foxp3^+CD4^+CD25^+$ cells in the total lymphocyte population.

1.8 Analysis of GITR

Whole blood treated with heparin sodium was diluted with RPMIl640 culture media at a 1:1 volume ratio. Blood was stimulated using phorbol ester (167 μg L^{-1}) and ionomycin (1 mg L^{-1}), and incubated in 50 mL L^{-1} CO_2 at 37°C for 6 h. After the above treatments, the whole blood obtained was used for measuring GITR. The Human regulatory T cell Staining kit used in our study was obtained from eBioscience, the RPMI1640 medium was from GIBCO, Phorbol ester and ionomycin were from Sigma, and calf serum was from Wuhan Sanli Biotechnology Company. A 50 μL sample of the whole blood obtained after stimulation was incubated with 10 μL of anti-CD3-PerCp and 5 μL of anti-CD8-APC-Cy7 mAbs in the dark at 4°C for 30 min. After haematolysis and washing with PBS, the resuspended cells were incubated in the dark at 4°C for 45 min with 10 μL anti-rat GITR-PE mAb (R&D Systems, Minneapolis, MN, USA) or 10 μL IgG1-PE (Beckman Coulter Inc) as an isotype-control. After washing, the cells were counted using FACSCanto and the data were analyzed using FACSDiva.

1.9 Histological examination

Livers were removed at the indicated time points and fixed in buffered 10% formalin. Four-micrometer paraffin-embedded sections were cut and stained with hematoxylin & eosin.

1.10 TNF-α measurement by enzyme-linked immunosorbent assay (ELISA)

Fresh liver tissue was prepared 7 days after transplantation. Tissue was lysed in buffer (25 mmol L^{-1} HEPES, pH 7.4, 0.1% CHAPS, 5 mmol L^{-1} $MgCl_2$, 1.3 mmol L^{-1} EDTA, 1.0 mmol L^{-1} EGTA) containing protease and phosphatase inhibitors. The presence of TNF-α in cleared lysates was measured by TNF-α ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, and adjusted to total protein concentration.

1.11 Assessment of spleen cytotoxic T lymphocyte (CTL)-mediated lytic activity by lactate dehydrogenase (LDH) release assay

A spleen leukomonocyte suspension was prepared for each group of rats. A tissue grinder was used to separate the cells in PBS. Then an LDH assay was used to analyze CTLs as previously described [9]. Briefly, splenic lymphocytes suspended in 10% FCS RPMI1640 culture media were added to a 96-well plate as CTL effector cells. B16 cells were added to the effector cells as target cells. The cells were cultured in an atmosphere of 5% CO_2 at 37°C for 24 h, then centrifuged at 1500 r min^{-1} for 5 min, and the cell-free-supernatant was retrieved for analysis of LDH levels. After termination of the experiment, 50 μL of the supernatant was drawn from each well of the 96-well plates and 50 μL lithium lactate (0.3 mol L^{-1}, pH 8.8) (Sigma-Aldrich Company, St. Louis, MO, USA) was added to each well and incubated in the dark at room temperature for 30 min. The absorbance of each well was determined to calculate the release of LDH. The lytic activity of CTLs was expressed in terms of the cytotoxicity of CTL to B16 and calculated using the following CTL formula [10,11]:

$$\text{CTL Lytic Activity} = \frac{\text{LDH released by CTL} - \text{the LDH idio-released by target cell}}{\text{maximum LDH released by target cell} - \text{the LDH idio-released by target cell}}$$

1.12 Statistics

Data are provided as mean±standard deviation. Kaplan-Meier survival graphs were constructed and Log rank test of the groups was used to calculate *P*-values. For comparison between groups, we used Chi-square test and Student-Newman-Keuls *q* test as appropriate and $P<0.05$ was considered statistically significant.

2 Results

2.1 Survival in the acute rejection group was lower than in the other groups

Survival of all rats in the control, homogeneity liver transplantation and idio-immunotolerance groups exceeded 100 d, and there was no significant difference in survival between the three groups. Survival of rats in the acute rejection group was 17–21 d, which was significantly lower than for the control group, the homogeneity liver transplantation group and the idio-immunotolerance group (P<0.05) (Figure 1).

2.2 Increased levels of ALT and TB were maintained in the acute rejection group

The ALT and TB of rats in the homogeneity liver transplantation group was increased on day 4, and recovered by day 14 after surgery. The ALT and TB of rats in the idio-immunotolerance group increased significantly (P<0.05) when compared with the control group and the homogeneity liver transplantation group on day 4 and day 7 after surgery. The ALT and TB of rats in the acute rejection group increased significantly when compared with the control group and the homogeneity liver transplantation group and continued to increase until day 14 (P<0.05) (Figure 2).

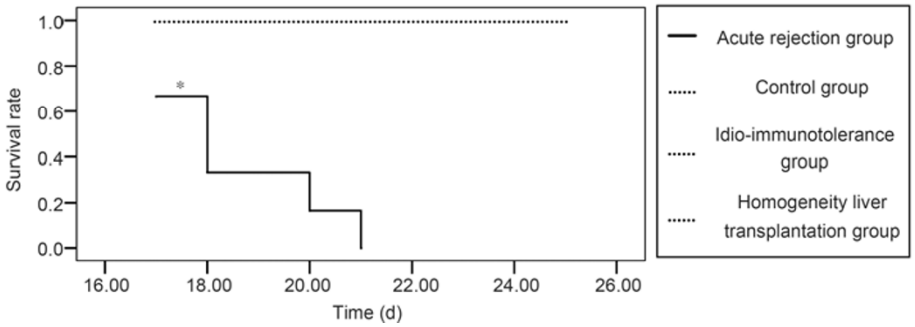


Figure 1 Kaplan-Meier survival curve. Survival rate of rats 16–26 days after operation. Survival of rats in the acute rejection group was significantly lower (*, P<0.05) than the other three groups.

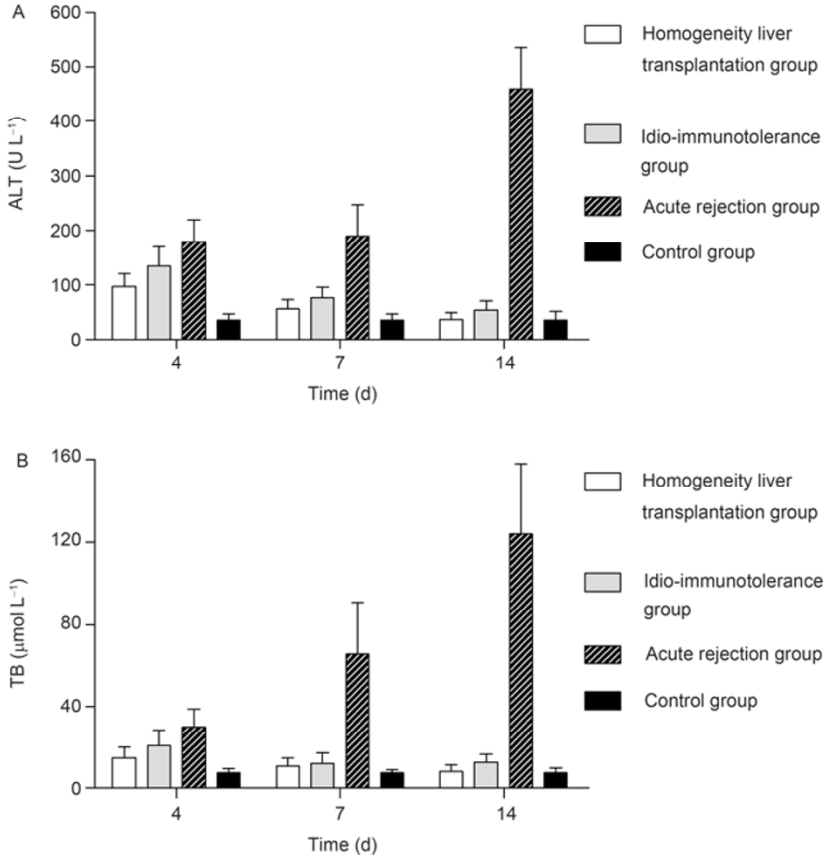


Figure 2 Analysis of alanine transaminase (ALT) and total bilirubin (TB). A, The ALT level of rats on days 4, 7 and 14 after operation. B, The TB level of rats on days 4, 7 and 14 after operation.

2.3 Changes in T cell subpopulations in the acute rejection group

There was no difference in the percentage of $CD3^+$ T cells between the transplant groups. Analysis of the T cell subpopulations by flow cytometry showed no difference in the $CD3^+CD4^+$ T cell counts between the four groups. The number of $CD3^+CD8^+$ T cells in the acute rejection group increased significantly (P<0.05) compared to the homogeneity liver transplantation, idio-immunotolerance group and control groups while the $CD4^+/CD8^+$ cell ratio decreased significantly (P<0.05) (Figure 3A).

The number of $Foxp3^+CD4^+CD25^+$ Tregs or $CD4^+CD25^+$ T cells in the acute rejection group was lower than in the control, homogeneity liver transplantation and idio-immunotolerance groups (P<0.05) (Figure 3B).

In the peripheral blood of rats, most lymphocytes were in the quiescent stage and GITR expression was not detectable on T cells [4,5]. After culture with phorbol ester and ionomycin, GITR expression on T cells increased in all groups. The GITR expression level on T cells from the acute rejection group was significantly higher than that in the other groups (P<0.05) (Figure 3C).

2.4 TNF-α levels in liver graft tissues were significantly higher in the acute rejection group

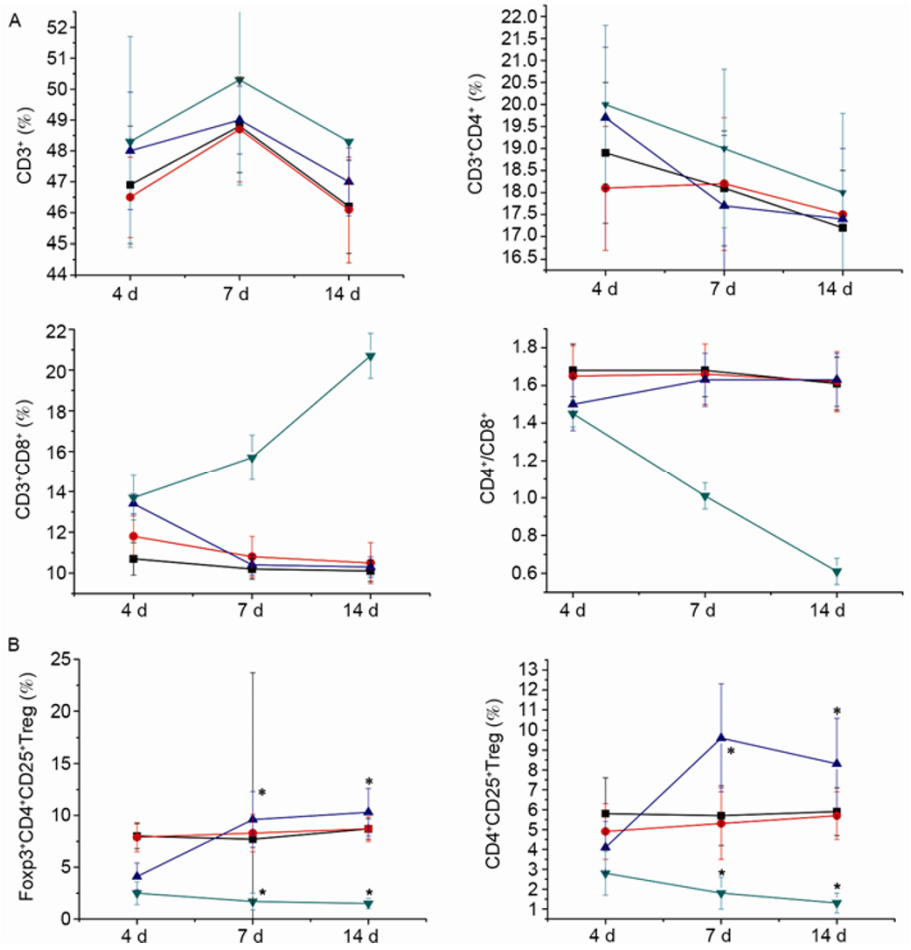
Hepatic levels of TNF-α in the acute rejection group were significantly higher compared to those in the control, homogeneity liver transplantation and idio-immunotolerance groups (P<0.05) (Figure 4).

2.5 CTL-mediated lytic activity in the acute rejection group increased continuously

CTL-mediated lytic activity in the spleen of recipients in the acute rejection group increased continuously. The spleen CTL-mediated lytic activity of the recipients in the acute rejection group increased by 32.6%, and was higher than those in the control, homogeneity liver transplantation and idio-immunotolerance groups (P<0.05) (Figure 3D).

2.6 Histological analysis showed typical changes of acute rejection only in the acute rejection group

On day 14 after liver transplantation, no significant difference was observed in the histology of the liver among the



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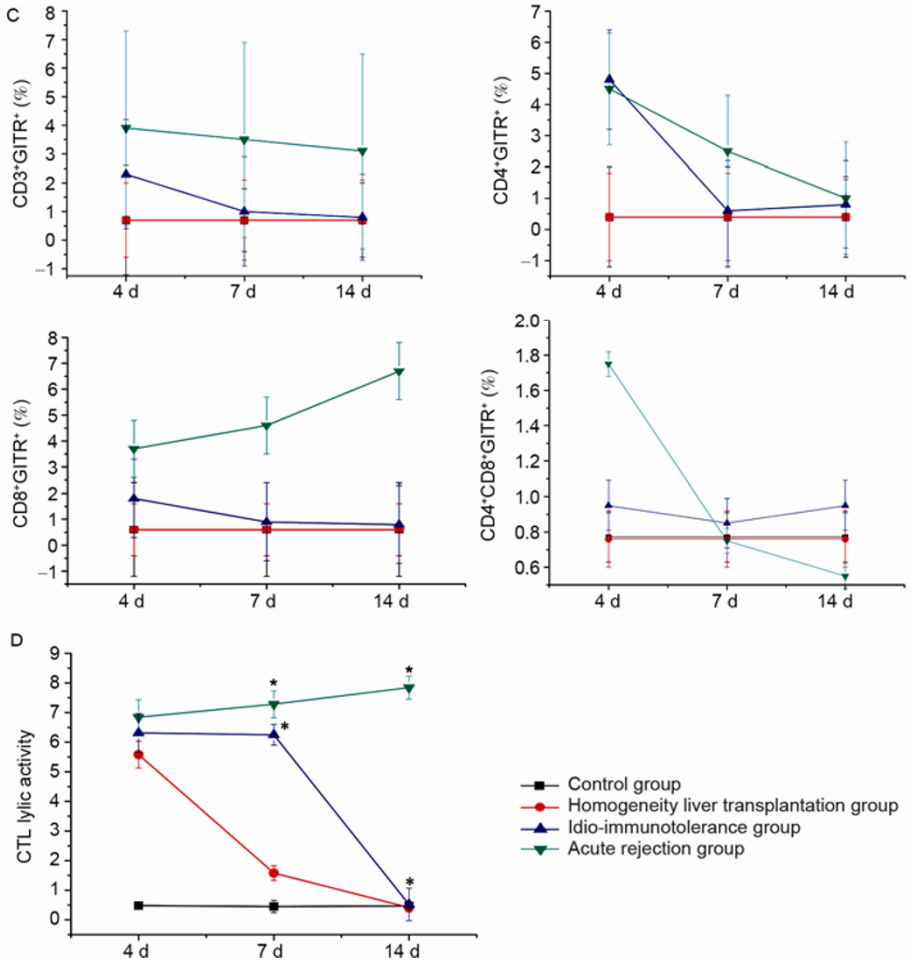

Figure 3 Detection of T cell subpopulations in the peripheral blood and CTL-mediated lytic activity. A, There was no difference in the percentage of $CD3^+$ T and $CD3^+CD4^+$ T cells between the transplant groups. The number of $CD3^+CD8^+$ T cells in the acute rejection group increased significantly (P<0.05), while the number of $CD4^+/CD8^+$ cells decreased significantly compared to the other groups (P<0.05). B, The ratio of $Foxp3^+CD4^+CD25^+$ Tregs and the expression of $CD4^+CD25^+$ T cells in the acute rejection group was significantly lower than those in the other groups (P<0.05). C, In the control group, GITR expression was not detectable on T cells. GITR expression on increased T cells from all groups after culture with phorbol ester and ionomycin. The GITR expression level of T cells in the acute rejection group was significantly higher than that in the other groups (P<0.05). D, The spleen CTL-mediated lytic activity of the recipients in the acute rejection group increased by 32.6%, and was significantly higher than those in the other three groups (P<0.05).

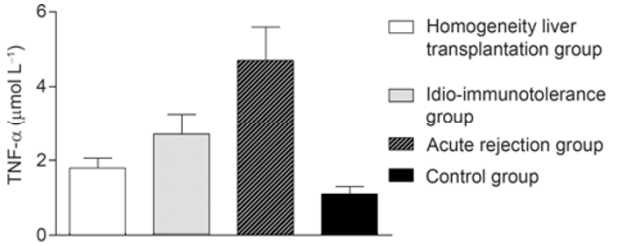

Figure 4 Hepatic levels of TNF-α in the acute rejection group were significantly higher compared to those in the control, homogeneity liver transplantation and idio-immunotolerance groups (P<0.05).

homogeneity liver transplantation, idio-immunotolerance and control groups (Figure 5A, B and D). However, in the acute rejection group, histological analysis showed changes typical of acute rejection including significant loss of hepatocytes, increased lymphocytic infiltration into the central-portal vein glisson, significant damage to the bile duct epithelium and coherence of inflammatory cells under the blood vessel endothelium (Figure 5C).

3 Discussion

Following allogeneic liver transplantation in animals such as rats and mice, idio-immunotolerance can be induced even when the transplant combination is fully major histocompatibility mismatched [12]. In our experiments, Lewis rats were used as donors and DA rats were used as recipients, and the latter survived for more than 100 d after surgery. However, serum ALT and TB levels significantly increased compared with the homogeneity liver transplantation group on days 4 and 7 after the transplant. There was no evidence of rejection as assessed by histological analysis of the hepatic grafts on day 14. These findings suggest that when

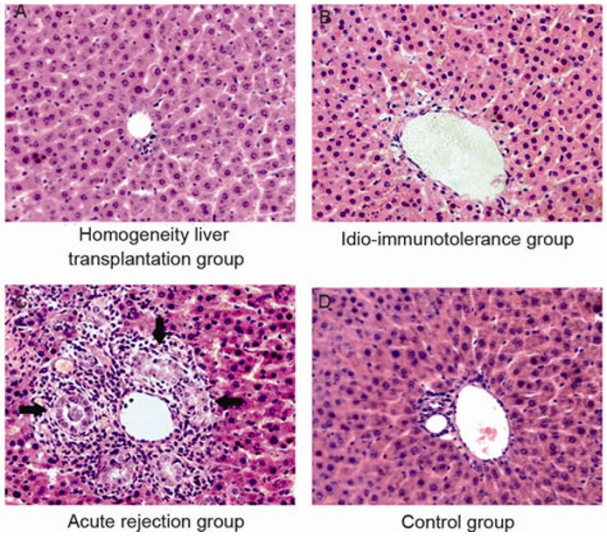

Figure 5 Histological analysis of the liver on day 14 post-transplantation. In the acute rejection group, histological analysis showed changes typical of acute rejection with significant loss of hepatocytes, increased lymphocytic infiltration into the central-portal vein glisson, significant damage to the bile duct epithelium and coherence of inflammatory cells under the blood vessel endothelium (arrowheads). No significant difference was observed in the liver histology of the other three groups.

Lewis rats were used as donors and DA rats as recipients, the hepatic graft suffered from rejection at an early stage after surgery but subsequently acquired idio-immunotolerance.

TBI preconditioning of the rat donors 24 h before transplantation eliminated lymphocytes in the liver and peripheral blood [7], thereby eliminating passenger lymphocytes in the graft. Our expression analysis showed an up-regulation of hepatic TNF-α mRNA after TBI preconditioning. Previous studies have reported that TNF-α levels increase in recipients during acute rejection after homogeneity organ transplantation such as liver transplantation [13]. Our results showed that TBI preconditioning adversely affected immunotolerance and induced acute rejection, suggesting lymphocytes in the hepatic graft play an important role in the induction of immunotolerance after liver transplantation.

Depletion of $CD25^+$ T cells in mice induces the development of autoimmune diseases [14], suggesting that a functional disturbance of $CD4^+CD25^+$ Treg also plays an important role in the pathogenesis of rejection following organ and/or cell transplantation [15,16]. Studies have also shown that $CD4^+CD25^+$ Tregs are associated with the induction of immunotolerance after liver transplantation [17,18]. In our study, we found no difference in the percentage of peripheral blood $CD3^+CD8^+$ T cells between the Control and the Homogeneity Liver Transplantation groups. However, compared with these groups, the percentage of peripheral blood $CD4^+CD8^+$ T cells in the idio-immunotolerance group, which were similar to the control group levels to begin with, increased on day 4 after surgery and continued to rise on day 7. In addition, $CD3^+CD8^+$ T cells down-regulated HLA-DR, a marker of T cell activation. The cytokine profile of $CD3^+CD8^+$ T cells tended toward Th2 polarization [19]. These data suggested that proliferation and activation of $CD3^+CD8^+$ T cells occurred in the acute rejection group. Our results also suggested that overactivation of $CD3^+CD8^+$ T cells in the acute rejection group was the result of an immunosuppression disorder mediated by $Foxp3^+CD4^+CD25^+$ Tregs and activation signals by GITR.

Piccirillo *et al.* [20] reported that $CD4^+CD25^+$ Tregs suppress the proliferation and activation of $CD3^+CD8^+$ T cells by suppressing the secretion of IL-2 and upregulation of CD25. The numbers of $Foxp3^+CD4^+CD25^+$ Tregs in the idio-immunotolerance group were significantly lower than those in the control and homogeneity liver transplantation groups from days 4 to 6, but returned to normal levels on day 14. This suggested that acute rejection after rat liver transplantation was a consequence of dyspoiesis of Tregs *in vivo* [21], which resulted in overactivation of $CD3^+CD8^+$ T cells. The number of $CD3^+CD8^+$ T cells in the idio-immunotolerance group also increased during the early stages after surgery.

Ronchetti *et al*. [22] reported that activation of GITR transforms $CD4^+CD25^+$ Tregs to responsive $CD4^+CD25^+$ T effector cells. Our study showed that the expression of GITR increased significantly in peripheral blood $CD3^+CD4^+$ T cells from the acute rejection group, and that the expression of GITR in peripheral blood $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in the acute rejection group was significantly higher than that in the control group. However, the number of the peripheral blood $CD3^+CD8^+$ T cells in the acute rejection group also increased. These observations suggested that although GITR signals provided costimulatory activation to T cells, $CD3^+CD8^+$ T cells might be more sensitive to costimulatory activation. Ronchetti *et al.* [22,23] reported that activated $GITR^{-/-}$ T cells were more susceptible to CD3-induced apoptosis than $GITR^{+/+}$ T cells, and that GITR signaling promoted the survival of T cells [24]. Thus, it is likely that the increase of GITR expressed by peripheral blood $CD3^+CD8^+$ T cells in the acute rejection group resulted in the prolonged survival and increased percentage of peripheral blood $CD3^+CD8^+$ T cells in the acute rejection group.

In summary, we found that GITR signaling induced proliferation, activation, and survival in the acute rejection group through costimulatory activation of lymphocytes, and inhibited apoptosis, ultimately resulting in a disorder of the peripheral blood T cell subgroup in the acute rejection group.

Abbreviations

CD	cluster of differentiation
Foxp3	forkhead box P3
GITR	glucocorticoid-induced tumor necrosis factor receptor
ALT	alanine aminotransferase

TB	total bilirubin
TBI	total body irradiation
Tregs	regulatory T cells
TNF-α	tumor necrosis factor-α
FITC	fluorescein isothiocyanate
APC	allophycocyanin
FACS	fluorescence activated cell sorte
PBS	phosphate buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol tetra-acetic acid
CTL	cytotoxic T lymphocyte
LDH	lactate dehydrogenase

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